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Genetic Differentiation between Ecological Two Types of the Japanese Firefly, *Hotaria parvula*: An Electrophoretic Analysis of Allozymes

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ABSTRACT—The Japanese firefly, *Hotaria parvula*, is separated into two types on the basis of body size (large and small type), flash interval of the mate-seeking male and geographic distribution pattern. The degree of genetic differentiation between the two types and between *H. parvula* and the congeneric species *H. tsushimana* was examined in 11 populations by allozyme analysis of 13 enzymes. The dendrogram for these populations constructed according to Nei's genetic distance showed that *H. parvula* was divided into two groups corresponding to small and large types. All the small-type populations were clustered within a slightly differentiated group, while the large type populations were considerably differentiated from one another. The genetic distance between the two types was 0.108, and that between *H. parvula* (of both types) and *H. tsushimana* was much higher (0.305) than that within either the large-type or small-type populations of *H. parvula*. These findings suggested that the small type of *H. parvula* originated from an ancestor similar to the large type of *H. parvula*.

INTRODUCTION

The firefly *Hotaria parvula* Kiesenwetter is widely distributed in Japan except in the Hokkaido and Okinawa Islands and is endemic to Japan. One of the present authors, Ohba, previously reported on the dimorphism in this species that males of large and small types were 10 and 5 mm in body length respectively [10, 11], and that this size dimorphism corresponded strictly to the dimorphism of flash interval of the mate-seeking male. The flash intervals of the large and small types were 1 and 0.5 sec respectively [11, 12]. Furthermore, the two types were distributed allopatrically. The large type was distributed in almost all the areas mentioned above, while the small type occurred in some parts of Kyushu, Shikoku and the western area of Honshu Islands [12]. These findings suggest that the two types are reproductively isolated from one another. Therefore, genetic analyses are urgently needed, in order to clarify this point.

Molecular approaches are powerful tools to elucidate the degree of genetic differentiation among populations. An electrophoretic technique of allozymes, in particular, has been widely used for population genetic studies [5, 6].

In this paper, we report the results of electrophoretic analysis of allozymes applied to ten populations of the two types of *H. parvula* and one population of *H. tsushimana* Nakane, which is a congeneric species closely related to the former.

We also construct a biochemical dendrogram from genetic distances calculated from allele frequencies and estimate a speciation process among them.

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MATERIALS AND METHODS

Fireflies

Fireflies used for electrophoresis were as follows: Large-type *Hotaria parvula* were collected from 1: Aomori, Aomori Prefecture (July 1989); 2: Tsukuba, Ibaraki Prefecture (July 1989); 3: Kamiyu, Mt. Hakone, Kanagawa Prefecture (July 1990); 4: Nagoya, Aichi Prefecture (May 1989); 5: Okazaki, Aichi Prefecture (June 1990); and 6: Toyonaka, Osaka Prefecture (May 1990). Small-type *H. parvula* were collected from 7: Yugawara, Kanagawa Prefecture (June 1989); 8: Ohiradai, Mt. Hakone, Kanagawa Prefecture (July 1990); 9: Kuma, Ehime Prefecture (July 1989); and 10: Kokura, Fukuoka Prefecture (June 1990). *Hotaria tsushimana* were collected from 11: Tsushima Island, Nagasaki Prefecture (June 1990). Specific localities are mapped in Figure 1, and the numbers of individuals used for electrophoresis in each population are shown in Table 1. Fireflies were stored at −20°C until used.

Fig. 1. A locality map of *Hotaria parvula* and *H. tsushimana* used in this study. Open and solid circles indicate collecting localities of the large- and small-types *H. parvula* respectively.

Electrophoresis

After wings were removed, whole bodies were homogenized individually with 250 μl of 20 mM phosphate buffer (pH 6.9) containing 1 mM EDTA in ice water bath. After centrifugation of the homogenate at 10000 rpm for 10 min at 4°C, 40% sucrose of the same volume as the clear supernatant was added, and 40 μl of this mixture was subjected to electrophoresis.

Electrophoresis was performed on 7.5% polyacrylamide gel according to Davis [3] using slab gel apparatus (gel size: 135×110×1 mm) for superoxide dismutase (SOD) and disc gel apparatus (column size: ϕ5×90 mm) for 12 enzymes as follows: asparate aminotransferase (AAT); aldolase (ALD); alkaline phosphatase (APH); α-glycerophosphate dehydrogenase (α-GPDH); glucose-6-phosphate dehydrogenase (G6PD); hexokinase (HK); lactate dehydrogenase (LDH); malate dehydrogenase (MDH); malic enzyme (ME); nothing dehydrogenase (NDH); phosphoglucomutase (PGM); and xanthine dehydrogenase (XDH). The buffer systems of running and stacking gel used were 0.38 M Tris-HCl (pH 8.9) and 0.03 M Phosphate-Tris buffer (pH 6.9) respectively. An electrode buffer was 0.38 M Glycine-Tris (pH 8.3). A constant current of 20 mA/gel for slab gel or 2.5 mA/column for disc gel was applied until the tracking dye (bromo phenol blue) migrated 5 cm. After electrophoresis, gels were stained for the 13 enzymes listed above. Staining mixtures for AAT, ME and SOD were prepared according to Marcus [7], Ayala *et al.* [1] and Beauchamp and Fridovich [2] respectively, and the staining mixture for the other enzymes was prepared according to Shaw and Prasad [14].

RESULTS

Figure 2 shows electrophoretic patterns of the 13 enzymes observed in ten populations of the two types of *H. parvula* and one population of *H. tsushimana*. Lanes L, S and T show zymograms in the large type and small type of *H. parvula* and *H. tsushimana* respectively. Table 1 shows allele frequencies of the 11 populations examined. Alleles are lettered beginning with *a*, in ascending order of zymogram mobility. In Figure 2, four enzymes (ALD, NDH, PGM and XDH) showed single-banded activity. G6PD, MDH, ME and HK

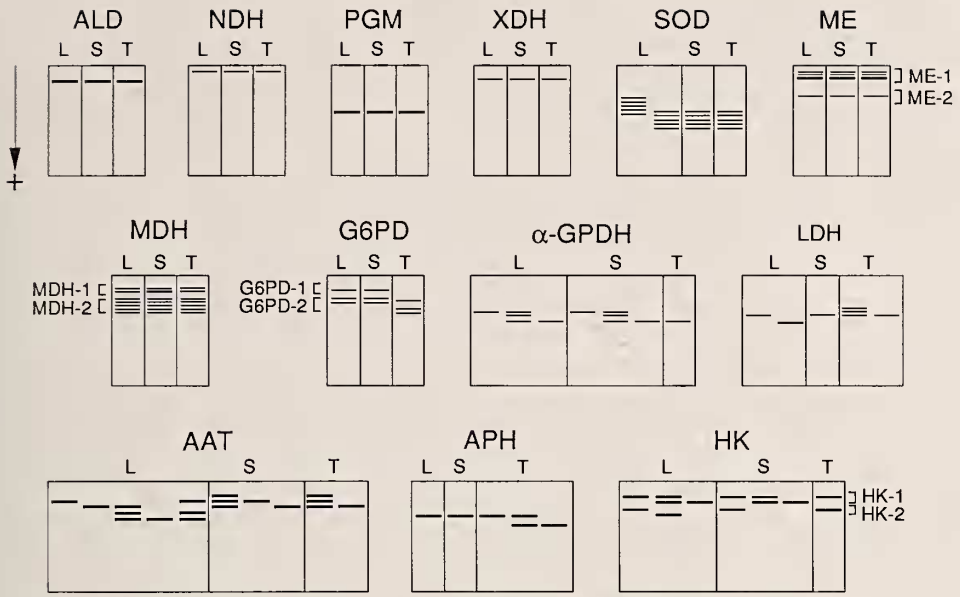

FIG. 2. Electrophoretic patterns of 13 enzymes of *Hotaria parvula* and *H. tsushimana*. Lanes L, S and T show zymograms in the large type and small type of *H. parvula* and *H. tsushimana* respectively.

showed several bands, and the gene loci shown in Figure 2 were presumed on the basis of their electrophoretic patterns. APH and HK-1 showed single- and double-banded phenotypes, and this variation was interpreted as a diallelic system cording for a monomeric protein at a single locus. In the Kuma population, no HK-1 zone was observed. At the HK-2 zone, electrophoretic mobility of *H. parvula* in the Kamiyu population was greater than that of other populations, and the alleles *b* and *a* were fixed in the Kamiyu and other populations respectively. α-GPDH and AAT showed single- and triple-banded phenotypes. These phenotypes were interpreted as polymorphism cording a dimeric protein at a single locus. LDH also exhibited single- and triple-banded phenotypes. Electrophoretic mobility of the single band in the Aomori population was greater than that of other populations, and the alleles *c* and *b* were fixed in the Aomori and other populations respectively. LDH activity in the Okazaki and Ohiradai populations was not detected. SOD showed two phenotypes. One showed five bands, which appeared in all populations. The other, appearing in the Nagoya, Okazaki and Toyonaka populations, also showed five bands, and a set of the bands shifted more slowly in mobility. The two phenotypes were interpreted as a manifestation of two different alleles at a single locus. As a result, the 17 gene loci coding for the 13 enzymes were presented.

In order to estimate genetic differentiation among populations, the Nei's genetic identity (I) and genetic distance (D) [8] were calculated using the GENDIST computer program of the PHYLIP Version 3.4 [4] from the gene frequencies data described on Table 1. The D value means the average number of codon differences per locus estimated from allele frequency data between populations. Table 2 shows I and D values among all pairs of the populations. The D values within the small-type populations ranged from 0.003 to 0.072 with a mean was 0.032, and those within the large type-populations ranged from 0.044 to 0.242 with a mean was 0.126. The average value of genetic distances between the two types of *H. parvula* was 0.108.

Figure 3 shows a biochemical dendrogram of the 11 populations constructed from D values by UPGMA (unweighted pair-group method with arithmetic mean) using the NEIGHBOR computer program of the PHYLIP Version 3.4 [4]. Solid and

Table 1. Allele frequencies at 17 loci coding for 13 enzymes of the two types of *Hotaria parvula* and *H. tsushimana* populations

Locus	Allele	large-type *parvula*						small-type *parula*				*tsushimana*
		1	2	3	4	5	6	7	8	9	10	11
ALD	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
APH	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.83
G6PD-1	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00
	b	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
G6PD-2	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00
	b	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
MDH-1	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MDH-2	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
HE-1	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
HE-2	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
NDH	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
PGM	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00
	b	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
XDH	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AAT	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.03	0.10
	b	1.00	0.12	0.90	1.00	1.00	0.09	0.00	0.00	0.00	0.33	0.00
	c	0.00	0.75	0.10	0.00	0.00	0.54	1.00	1.00	0.88	0.64	0.90
	d	0.00	0.13	0.00	0.00	0.00	0.37	0.00	0.00	0.00	0.00	0.00
α-GPDH	a	1.00	1.00	0.00	0.18	0.50	0.00	0.18	0.20	0.00	1.00	0.00
	b	0.00	0.00	1.00	0.82	0.50	1.00	0.82	0.80	1.00	0.00	1.00
HK-1	a	1.00	1.00	1.00	1.00	0.58	0.81	1.00	0.70	—	0.60	1.00
	b	0.00	0.00	0.00	0.00	0.42	0.19	0.00	0.30	—	0.40	0.00
HK-2	a	1.00	1.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	b	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LDH	a	0.00	0.00	0.00	0.00	—	0.00	0.00	—	0.00	0.00	0.03
	b	0.00	1.00	1.00	1.00	—	1.00	1.00	—	1.00	1.00	0.97
	c	1.00	0.00	0.00	0.00	—	0.00	0.00	—	0.00	0.00	0.00
SOD	a	0.00	0.00	0.00	0.87	0.83	1.00	0.00	0.00	0.00	0.00	0.00
	b	1.00	1.00	1.00	0.13	0.17	0.00	1.00	1.00	1.00	1.00	1.00
N		8	8	10	10	11	6	8	16	8	15	14

N indicates the number of individuals assayed. Population numbers correspond as follows:
1: Aomori; 2: Tsukuba; 3: Kamiyu, 4: Nagoya; 5: Okazaki; 6: Toyonaka; 7: Yugawara; 8: Ohiradai; 9: Kuma; 10: Kokura; 11: Tsushima.

open circles of branch ends in the dendrogram indicate the small- and large-type populations of *H. parvula* respectively. As shown in the dendrogram, *H. parvula* was divided genetically into two groups corresponding to the small and large types. The only exception was that theTsukuba population was included in the small-type group, though its morphological characters represented the large type. In the large-type group, Nagoya, Okazaki and Toyonaka populations were the first to group, after which the Kamiyu and Aomori populations joined to them, in that order. *H. tsushimana* was apparently differentiated from *H. parvula*, and the average value of genetic distances between them was 0.305.

DISCUSSION

The average genetic distance within the small-type populations was 0.032. This was a reasonable value for a local population level when compared with similar studies previously reported [5, 9].

TABLE 2. Genetic identity (above diagonal) and genetic distance (below diagonal) among ten populations of *Hotria parvula* and one population of *H. tsushimana*

Population	large-type *parvula*						small-type *parvula*				*tsushimana*
	1	2	3	4	5	6	7	8	9	10	11
1. Aomori	—	0.900	0.822	0.854	0.928	0.779	0.841	0.889	0.817	0.904	0.647
2. Tsukuba	0.105	—	0.848	0.872	0.882	0.872	0.956	0.949	0.934	0.988	0.754
3. Kamiyu	0.196	0.163	—	0.891	0.861	0.846	0.890	0.875	0.891	0.850	0.714
4. Nagoya	0.156	0.136	0.114	—	0.981	0.956	0.894	0.879	0.892	0.875	0.713
5. Okazaki	0.074	0.125	0.149	0.018	—	0.935	0.871	0.880	0.934	0.908	0.666
6. Toyonaka	0.249	0.136	0.166	0.044	0.066	—	0.924	0.919	0.927	0.866	0.741
7. Yugawara	0.172	0.044	0.116	0.111	0.137	0.078	—	0.994	0.997	0.942	0.814
8. Ohiradai	0.117	0.051	0.133	0.128	0.127	0.084	0.005	—	0.996	0.949	0.794
9. Kuma	0.201	0.068	0.115	0.113	0.067	0.075	0.003	0.003	—	0.930	0.806
10. Kokura	0.100	0.012	0.162	0.132	0.096	0.143	0.059	0.052	0.072	—	0.736
11. Tsushima	0.435	0.281	0.336	0.338	0.406	0.298	0.204	0.230	0.215	0.305	—

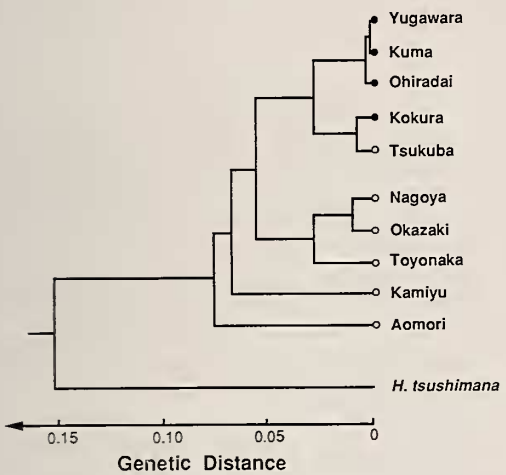

FIG. 3. A biochemical dendrogram of ten populations of *Hotaria parvula* and one population of *H. tsushimana* constructed from Nei's genetic distance by UPGMA. Open and solid circles of branch ends indicate the large- and small-type populations of *H. parvula* respectively.

Within the large-type populations, the average genetic distance was 0.126. The large-type populations were genetically more differentiated from one another than were the small ones. Namely, Aomori, Kamiyu and Toyonaka populations have specific alleles fixed at LDH,HK-1 and SOD loci respectively.

The average genetic distance between the two types of *H. parvula* was 0.108, which was a comparable value in a local population or on a subspecies level [5, 9]. At AAT locus, the allele frequency differed in the two types. Allele *b* was dominant in all the large-type populations except Tsukuba and Toyonaka, while allele *c* was dominant in the small-type populations. But this locus was insufficient to distinguish the two types. In the 17 loci assayed, no diagnostic alleles to identify the two types were detected. In spite of only slight genetic differentiation evident between the two types, they are distinguishable by other criteria. Body size of the two types is apparently different (10 and 5 mm in male) [10, 11], and this corresponds to a difference in flash interval of the mate-seeking male. These intervals are 1 and 0.5 sec in the large and small types respectively [11, 12]. Furthermore, the two types are distributed allopatrically. The large type is distributed in almost all areas of Japan except the Hokkaido and Okinawa Islands, while the small type occurs in some parts of Kyushu, Shikoku and in the western area of Honshu Islands [12]. At Mt. Hakone (Kamiyu and Ohiradai populations), the large type occupies higher altitudes, and the small type lower altitudes, and the boundary between them was at an altitude of 700 to 900 m [10, 11]. Thus, their allopatry is appar-

ent. Allozymic results also indicated that gene flow between the two contacted populations at Mt. Hakone was restricted because alleles *b* and *a* at the HK-2 locus were fixed in the Kamiyu and Ohiradai populations respectively. Moreover, Ohba and Goto have reported with regard to an artificial cross-mating experiment involving the two types that they copulated reciprocally, but that they separated only in a minute [13]. In our observations, continuation time of copulation was more than one hour in *Hotaria*. This strongly suggests that the two types are reproductively isolated.

Generally, reproductive isolation is the criterion used to assign biological species. Two newly-born species are expected to have reproductive isolation and occur allopatrically like this. Based on the morphological, ecological, genetic and cross-mating results described above, we consider the degree of differentiation between the two types of *Hotaria parvula* to correspond to two different species, despite the short genetic distance.

Of the 17 loci studied, three diagnositc loci (G6PD-1, G6PD-2 and PGM) were specific to distinguish *H. tsushimana* from *H. parvula*. The average value of genetic distance between one population of *H. tsushimana* and ten populations of *H. parvula* (including the two types) was 0.305. As shown in the dendrogram, the degree of genetic differentiation between *H. tsushimana* and *H. parvula* was much higher than that within either the large-type or small-type populations of *H. parvula*. The two species are also distinguishable on the basis of color pattern. The pronotum of *H. parvula* is red with a black speckle, while that of *H. tsushimna* is yellowish orange without speckle. On the other hand, male body size (8 mm) and flash interval of the mate-seeking male (1 sec) in *H. tsushimana* [11] are more similar to those of the large type of *H. parvula* than to the small ones, and the dendrogram shows that *H. tsushimana* is genetically originated. Thus, these results lead to the speculation that ancestral forms of *Hotaria* are similar to *tsushimana* and the large type of *parvula*.

A dendrogram constructed from genetic distances contains information important not only to phylogenetic relationships but also to the speciation process. Furthermore, the D value is correlated to the divergence time from a common ancestor [9]. According to Nei's equation [9], the dendrogram showed that *Hotaria* diverged into two lineages (*tsushimana* and *parvula*) about 1.5 million years ago. Thereafter, *parvula* lineage diverged into large and small types about 0.5 million years ago. Consequently, the dendrogram suggests that the small type of *H. parvula* speciated from an ancestor similar to the large type of *H. parvula*.

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